

Title: Maternal prenatal stress and placental gene expression of *NR3C1* and *HSD11B2*; the effects of maternal ethnicity

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Summary

Background: Prenatal stress is associated with altered fetal and infant development. Previous studies have suggested that these effects may be mediated in part via altered functioning of placental enzymes and receptors involved in the HPA-axis, including the glucocorticoid receptor (*NR3C1*) and *HSD11B2*, the enzyme which metabolises cortisol. However, previous studies have not examined the potential ethnicity effects on these associations. This study aimed to characterise the association between maternal prenatal stress and placental genes expression and subsequently, any potential effect of maternal ethnicity.

Method: Pregnant women(n=83) were recruited prior to elective caesarean section and assessed for trait anxiety, depression and life events. Placentas were collected and placental gene expression of *NR3C1* and *HSD11B2* were analysed. We examined associations between maternal prenatal stress and placental gene expression, and the tested for a possible moderating effect of maternal ethnicity(59.0% Caucasian;41.0% non-Caucasian:12.0% South Asian;6.0% African/African-American;14.4% Other;8.4% Mixed).

Results: Analyses demonstrated a trend in the association between both maternal trait anxiety and depression symptoms with placental gene expression of *NR3C1*(adj. β =.220,p=.067;adj. β = .212,p=.064 respectively). We found a significant interaction with maternal ethnicity(β =.249;p=.033). In Caucasian women only prenatal trait anxiety and depressive symptoms were associated with an increase in placental *NR3C1* expression(adj. β =.389,p=.010;adj. β =.294;p=.047 respectively). Prenatal life

events were associated with a down regulation of
HSD11B2(adj. β =.381;p=.008), but only in Caucasians.

Conclusion: These results support previous findings of an association between maternal prenatal stress and the expression of placental genes associated with the HPA-axis, but only in Caucasians. These ethnic specific findings are novel and require replication in different populations.

Key words: Prenatal, Depression, Anxiety, Placenta and Ethnicity

1. Introduction

There is good evidence that maternal prenatal stress, including anxiety, depression and stressful life events, is associated with an increased risk of altered behavioural, cognitive and emotional development of the child (Glover et al., 2015; O'Connor et al., 2007; O'Connor et al., 2003). These changes in child development are, in turn, indicators of vulnerability for the development of psychopathology later in life (Schlotz and Phillips, 2009), with an increase in psychiatric risk during later childhood and adolescence following exposure to maternal prenatal distress (Capron et al., 2015; O'Donnell et al., 2014; Pawlby et al., 2011; Pearson et al., 2013). However, the underlying biological changes that may mediate the associations between maternal prenatal mood and child outcome are not well understood.

One potential mechanism is an alteration in the metabolism of cortisol by the placenta. Cortisol is known to cross the placenta, to some degree, and the correlation between maternal plasma and amniotic fluid cortisol has been found to be greater in more anxious mothers, suggesting an increased permeability to cortisol associated with raised anxiety (Glover et al., 2009). In animal studies, fetal exposure to raised levels of cortisol has been associated with a wide range of effects in offspring, including a reduction in birth weight, increase in adult blood pressure, and an increase in anxiety behaviours (Seckl and Holmes, 2007). In humans, fetal exposure to increased cortisol has been associated with a decrease in child cognitive function (Bergman et al., 2010).

The placenta has high levels of corticosteroid 11-beta-dehydrogenase 2 (*HSD11B2*), the enzyme that converts cortisol into inactive cortisone. This enzyme is located within the syncytiotrophoblast during the second and third trimester of human pregnancy (Pepe et al., 1999; Schoof et al., 2001). Studies in rodents have shown that exposure to maternal prenatal stress is associated with a decrease in the gene expression of placental *HSD11B2* (Jensen Peña et al., 2012; Mairesse et al., 2007). Some human studies have also shown a down-regulation of *HSD11B2* gene expression and reduction of enzymatic activity in the placentae of mothers with higher levels of prenatal anxiety (O'Donnell et al., 2012). This finding has been replicated in an Australian birth cohort, where high anxiety and depressive symptom scores at 28-34 weeks gestation were associated with a reduction in placental *HSD11B2* gene expression (Seth et al., 2015). Monk et al. (2016) have found an association between maternal prenatal distress, placental methylation of *HSD11B2* and altered fetal behaviour (Monk et al., 2016). However, a study by Reynolds et al. reported no association between maternal depressive symptoms and placental gene expression of *HSD11B2* (Reynolds et al., 2015).

Maternal prenatal depression has been associated with an increase in the expression of the glucocorticoid receptor (*NR3C1*) gene in the placenta in several studies (Conradt et al., 2013; Mina et al., 2015; Reynolds et al., 2015). *NR3C1* is a nuclear receptor which functions both as a transcription factor and as a transcription factor regulator (Palma-Gudiel et al., 2015). *NR3C1* has been shown to be expressed in placental tissue and although its function is unclear, it has been postulated that it may be an upstream regulator of placental *HSD11B2* gene expression (Garbrecht and Schmidt, 2013). Thus an increase in placental *NR3C1* gene expression may be a

mechanism through which the placenta increases its sensitivity to glucocorticoids and fetal glucocorticoid exposure (Seckl and Holmes, 2007).

Thus previous research has shown that a range of different types of prenatal stress, including symptoms of anxiety, depression as well as life events, are associated with increased risk of altered outcome for the child including psychopathology (Glover et al., 2014; Kinsella and Monk, 2009). Prenatal symptoms of both anxiety and depression have also been found to be associated with altered expression of HPA-axis associated genes in the placenta. The majority of this research has been conducted in predominantly Caucasian populations and therefore, it is currently unclear if there are different associations seen in different populations. It is important to study both Caucasian and non-Caucasian populations. The current study was designed to investigate the associations between maternal prenatal depressive symptoms, anxiety symptoms and life events with placental gene expression of *NR3C1* and *HSD2B11*, and includes subjects with different ethnicities.

2. Methods

2.1. Participants

Study participants (n = 83) were recruited into the My Baby and Me (MBAM) Study the day before elective caesarean section at Queen Charlotte's and Chelsea Hospital, West London. Women with singleton pregnancies, with no known obstetric medical disorders and fluent in English were approached for this study. Within the MBAM Study, 140 women were approached, 95 women consented, 55 women declined. Of the 95 women who consented, 90 placentae samples were obtained (3 participants withdrew from the study, 2 participants were withdrawn as they were no longer eligible for the study). Women who took glucocorticoid medication during their pregnancy were excluded from the study. Ethical approval for the study was obtained from the National Research Ethics Service (London, Chelsea; REC Ref – 13/LO/1436).

2.2. Maternal Questionnaires

Mothers completed the following self-rating questionnaires shortly after recruitment.

The Edinburgh Postnatal Depression Scale (EPDS), a widely used and well validated questionnaire was used to assess symptoms of maternal depression (Cox et al., 1996; Cox et al., 1987). It has 10 questions with higher scores reflecting increased levels of depressive symptoms.

The Spielberger Trait Anxiety Inventory (STAI) is comprised of 20-questions and is a widely used and well-validated questionnaire to assess symptoms of anxiety. The

STAI-Trait Anxiety sub-scale examines how a participant generally feels and higher scores indicate elevated maternal anxiety.

The Life Events Questionnaire (LEQ) adapted from Barnett et al. (1983) is a 26-item maternal report of perceived stress relating to life event(s) during the course of pregnancy (Bergman et al., 2007).

2.3. Obstetric and maternal demographic characteristics

Information on maternal age, ethnicity, smoking (smoking vs. non-smoking), alcohol consumption (unit intake per week), prescription drug use, clinical indication for elective caesarean section, gestational age, and parity were collected at recruitment. Data on birth outcomes and infant sex were collected from hospital notes following delivery.

2.4. Placental collection

Healthy, intact placentas were collected immediately following delivery and dissected within 60 minutes. The umbilical cord was removed and the placental weight recorded. Tissue samples were taken from the maternal side of the placenta, at 5 random sampling sites. To ensure that the placental tissue was fetal in origin, the uppermost cotyledon surface was removed and trophoblast tissue taken from the central part of the placenta to include samples of the syncytiotrophoblastic region. The 5 samples were dissected in ice cold phosphate buffered saline (PBS), and pooled to control for intra-placental variation in gene expression. Samples were stored at -80 °C until required.

2.5. RNA Preparation

Total RNA was extracted from placental trophoblastic tissue using RNeasy Mini Kits (Qiagen, Crawley, UK), following the manufacturers protocol. RNA quantity was

assessed using a NanoDrop ND-1000 spectrophotometer (75–220 nm), whilst RNA integrity was examined using a Bioanalyzer 2100 (Agilent, Stockport, UK). Following previous recommendations, samples with an RNA integrity number ≥ 5 were considered of sufficient quality for subsequent use in cDNA synthesis and RT-PCR (Fleige and Pfaffl, 2006).

2.6. Reverse transcriptase PCR

cDNA was synthesised from RNA (2ng/16µl) using the Superscript II system as per the manufacturer's instructions (Invitrogen, Paisley, UK). Genomic DNA was removed from RNA samples using a DNase treatment (DNase I, Invitrogen). Wells contained 4.0µl of KiCqStart® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, Dorset, UK), 2.6µl of DEPC-treated water and 0.4µl of appropriate forward and reverse primers, each at a concentration of 800nM. Non-template controls were run with 0.6µl of RNase free water to check for contamination, whilst 0.6µl of sample cDNA was added to all other wells, giving a total reaction volume of 8µl per well/reaction.

Placenta *HSD11B2* and *NR3C1* gene expression analyses were assayed in duplicate and normalised to the geometric mean of ribosomal protein L19 and 14-3-3 protein YWHAZ using the following primer sequences: *HSD11B2* forward:

CTACTCATGGACACATTCAGCT, reverse: TCACTGACTCTGTCTTGAAGC; *NR3C1* forward:

CGACCAATGTAAACACATGCT, reverse: CCGTCCTTAGGAACTGAAGAG; L19 forward:

ATTCTTCGTGTTACTACAGCTGAC, reverse: CATTCTAAGGATGAAAGTAGCTC; YWHAZ

forward: GATGACAAGAAAGGGATTGTCTG, reverse: CAGTCTGATAGGATGTGTTGGT.

There was a strong correlation between the two housekeeping genes ($R_s = .597$, $p < .001$).

RT-PCR was performed using a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Cycling parameters were: (1) 2 min at 95 °C, (2) 5 sec at 94 °C and (3) 15 sec at 65 °C, with steps 2–3 being repeated for 40 cycles. The semi-quantitative assay provides information on the level of expression of the gene of interest and RT-PCR data is presented as as cycle threshold (Δ Ct) values (Schmittgen and Livak, 2008).

2.7. Data Analysis

All statistical tests were performed using SPSS (v23.0). The placenta gene expression data was not normally distributed, so non-parametric statistical tests were used to analyse the data. Associations between maternal prenatal stress exposures (prenatal trait anxiety symptoms, depressive symptoms and life events) and the placental gene expression of *HSD11B2* and *NR3C1* were analysed using linear regression. Analyses were adjusted for covariates including maternal educational status, maternal prenatal smoking, and placental weight. These covariates were selected as they were significantly associated with either predictor or outcome variables. Maternal household income was not used as a covariate as it was not significantly associated with either the predictor or outcome variables. Previous research in this field has predominantly been conducted in Caucasian populations. Therefore, to test the effect of ethnicity within this sample, interaction analyses were conducted. For completeness, the effect of infant sex was also assessed using interaction analyses, but no significant interaction effects were observed. All variables included in the interaction terms were centred prior to analysis.

To ease interpretation gene expression values (expressed as cycle threshold (ΔCt) values) were inverted [$x(-1)$], such that a higher value represents an increase in gene expression. In addition, outliers, which fell outside the mean Ct value $\pm 3SD$'s, were excluded from analyses (Number of outliers: *HSD11B2* analyses=1; *NR3C1* analyses=3). Furthermore, placental weight for one participant was not obtained at the time of birth and this participant was therefore not included in the regression models.

3. Results

The demographics and psychometric data for the participant group with placental gene expression data are shown in Table 1. The majority of participants had high levels of education, as is typical for the population from this hospital. With regard to maternal ethnicity, 59.0% were Caucasian; 12.0% South Asian; 6.0% African/African-American; 14.4% other and 8.4% mixed ethnic groups. There was no significant difference between the Caucasian and non-Caucasian groups with regard to these demographic and psychometric variables.

Table 1 here

We found a trend in the association between placental *NR3C1* gene expression and maternal prenatal trait anxiety symptoms ($\text{adj.}\beta = .211$; $p = .076$). Maternal prenatal depressive symptoms was also weakly associated with placental *NR3C1* gene expression ($\text{adj.}\beta = .212$; $p = .064$). There was no evidence for an association between other measures of maternal prenatal stress and placental gene expression in the whole cohort (see Table 2).

Table 2 here

Previous research has been conducted in predominantly Caucasian populations and as this cohort was ethnically diverse, interaction analyses were undertaken to examine for a potential moderating effect of maternal ethnicity on the association between maternal prenatal stress and placental gene expression. There was a significant interaction between maternal prenatal trait anxiety symptoms and maternal ethnicity with respect to the placental gene expression of *NR3C1* (Interaction analysis; $\text{adj.}\beta = .249$, $p = .033$). Once the data was split by ethnicity, maternal trait anxiety symptoms was shown to be a significant positive predictor of *NR3C1* gene expression in the Caucasian population ($\text{adj.}\beta = .389$, $p = .010$) suggesting that higher anxiety symptoms in these mothers predicts an increase in placental *NR3C1* gene expression (Figure 1A). However, there was no association seen between maternal prenatal trait anxiety symptoms and placental gene expression of *NR3C1* in the non-Caucasian mothers ($\text{adj.}\beta = .091$, $p = .673$). Although there was no significant interaction between maternal prenatal depressive symptoms and maternal ethnicity with placental *NR3C1* gene expression (interaction analysis; $\beta = .133$; $p = .233$), there was an association between maternal prenatal depressive symptoms and placental gene expression of *NR3C1* in Caucasian women ($\text{adj.}\beta = .294$, $p = .047$; Figure 1B). No such association was observed in non-Caucasians ($\text{adj.}\beta = .176$, $p = .382$).

There was a significant interaction between maternal prenatal life events and maternal ethnicity with the placental gene expression of *HSD11B2* (interaction analysis; $\text{adj.}\beta = -.385$, $p = .020$). Once split by ethnicity, there was a negative association between the number of prenatal life events in pregnancy and placental gene expression of *HSD11B2* in the Caucasian population ($\text{adj.}\beta = -.381$, $p = .008$;

Figure 1C). This association was not observed in the non-Caucasian population (adj. β = .140, p = .445).

To further explore which sub-group within the non-Caucasian group was driving these associations, correlations were conducted and suggest that these findings may be led by results from women who identified as being South Asian (Supplementary analyses 1)

Figure 1 here.

4. Discussion

This study showed a positive trend in the association between maternal prenatal anxiety and depressive symptoms with placental gene expression of *NR3C1* in the whole cohort. No consistent evidence was found of an association between maternal prenatal anxiety and depressive symptoms and placental *HSD11B2* gene expression. However, interaction analyses by ethnicity demonstrated marked differences between Caucasian and non-Caucasian mothers. In Caucasian participants, prenatal trait anxiety symptoms were predictive of an increase in placental *NR3C1* gene expression, and prenatal life events were predictive of a decrease in *HSD11B2* placental gene expression. No such associations were observed in non-Caucasian women. Thus in the Caucasian women the study provides further evidence of an association between prenatal stress and expression of these placental genes associated with the HPA-axis, in the same direction as previous studies.

There are several strengths to this study. Firstly, the study sample contained placental samples from an ethnically diverse population resulting in a novel focus. Previous research in this field has predominately involved Caucasian populations. Secondly, the placental samples were all collected and stored with one hour of delivery, which reduced the risk of tissue degradation. They were also obtained from subjects who had an elective caesarean, eliminating any possible effects of labour on gene expression (Cindrova-Davies et al., 2007). Finally, the gene expression within the placental tissue was calculated using the ΔCt method that corrects for internal variation between the placentae by adjusting for well-regulated and stable housekeeping genes (YWHAZ and L19) (Meller et al., 2005; Patel et al., 2002).

There were also a number of limitations to consider. The study population was modest in size with small numbers of women from a range of different non-Caucasian ethnic groups. The non-Caucasians were predominantly from the Indian subcontinent, but numbers from each sub-group were too small for further analysis, and as these are quite varied ethnic groups it is difficult to draw any firm conclusions about them. It remains possible that the differences observed between the Caucasians and the others are due to social rather than biological factors. They may have experienced past trauma or racism, for example, which may have altered their resilience. However, as Table 1 shows there were no significant differences in any of the demographic or psychometric parameters studied between the two groups. Further analyses of the sub-groups within the non-Caucasian population suggest that these findings may be driven by those who identify as South Asian. However, due to the small size of the subgroup ($n=10$) it is difficult to extrapolate from these findings and further analyses within larger cohorts would be needed to examine these associations further. As the levels of prenatal stress were collected via self-report questionnaires, some caution is warranted, and they clearly do not represent clinical diagnoses. Additionally, there was only one-time point at which maternal prenatal anxiety and depression scores were assessed and this was at the end of pregnancy. As the participants completed their questionnaires prior to elective caesarean section this may have resulted in elevated scores, especially with regard to anxiety. We do not know how long such anxiety may have been raised, or how long changes in the gene expression take to occur. There was a lack of consistency in the findings with different maternal psychometric measures. As anxiety and depressive symptoms are highly co-morbid one might have expected a larger degree of overlap within findings with

different but related predictors. We currently do not have enough evidence to determine whether symptoms of anxiety, depression or life event stress actually have a differential effect on the expression of the genes studied.

When we consider our findings in the context of the existing scientific literature, we note that our findings support previous research that has shown associations between maternal stress in pregnancy and upregulation of placental *NR3C1* gene expression (Mina et al., 2015). Previous studies have also shown associations between antenatal depressive symptoms and increased DNA methylation of *NR3C1* and BDNF in infant buccal cells and in cord blood following exposure to antenatal depression (Braithwaite et al., 2015; Hompes et al., 2013; Oberlander et al., 2008). This increase in methylation suggests a reduction in gene expression of *NR3C1* in the offspring and may be the result of overexposure to glucocorticoids in pregnancy through increased placental transmission of glucocorticoids.

Maternal antenatal trait anxiety has previously been associated with a down regulation of placenta *HSD11B2* gene expression (O'Donnell et al., 2012; Seth et al., 2015). Indirect evidence in human studies has shown that there is a correlation between cortisol concentrations in maternal plasma and amniotic fluid (assessed at the point of amniocentesis) in highly anxious women when compared to non-anxious women (Glover et al., 2009). This suggests that there may be greater permeability of the fetal compartment to maternal cortisol levels in pregnancies exposed to higher levels of prenatal anxiety. However, the evidence to support the association between elevated maternal prenatal anxiety and depressive symptoms and placental *HSD11B2* gene expression is mixed as a study by Reynolds et al. (2015) did not show

an association between maternal depression in pregnancy and placental gene expression of *HSD11B2* (Reynolds et al., 2015). In the Caucasian population only, we have shown a down regulation of placental *HSD11B2* associated with an increased number of life events experienced during pregnancy. This has not previously been demonstrated. It will only be possible to resolve whether there are indeed differential effects between anxiety, depression and life events on placental gene expression with a much larger sample size.

The majority of the current literature has been focused on populations, which are all, or predominantly, Caucasian in origin. The findings of the current study raise an interesting question, as to whether women from different ethnicities vary in their placental response to prenatal stress and furthermore whether any such difference might result in changes to infant and child outcome associated with exposure to prenatal stress.

There have been few studies that have examined the effect of maternal prenatal stress in non-Caucasian populations. The studies that there have been, span a wide range of ethnic populations and stressors and mostly focus on birth outcomes of the infant, such as birth weight and birth length. These studies suggest that maternal exposure to prenatal stressors such as common mental health disorders, war, or physical violence can be associated with a reduction in birth weight (Ferri et al., 2007; Mulligan et al., 2012; Nasreen et al., 2010; Niemi et al., 2013; Patel and Prince, 2006; Rahman et al., 2007), reduction in birth length (Broekman et al., 2014), an increased risk of pre-term birth (Niemi et al., 2013), greater risk of fetal distress during delivery (Zahran et al., 2010), increased time in labour (Hanlon et al., 2009), delays in breastfeeding initiation (Hanlon et al., 2009) and admission to neonatal

intensive care (Chung et al., 2001). However, these studies did not examine the effects of these maternal stressors on offspring outcome with respect to maternal ethnicity. No studies to date have examined placental gene expression across different ethnic groups. One study in a non-Caucasian population demonstrated an association between maternal prenatal war exposure and the methylation status of NR3C1 in the cord blood of infants within a cohort from the Democratic Republic of Congo (Mulligan et al., 2012). These findings are similar to that of a study conducted in a Caucasian population, but this study was conducted on women experiencing prenatal depression rather than acute prenatal stress due to war exposure (Oberlander et al., 2008). Further studies are necessary to assess if there is any different effect of these maternal stressors on offspring outcome dependent on maternal ethnicity.

In future studies it will of interest, as well as confirming the differences in placental expression in different ethnic groups, to determine whether this is associated with differences in child outcome.

4.1. Conclusion

This study provides further evidence that maternal prenatal stress is associated with changes in the placental gene expression of an enzyme and receptor involved in the HPA-axis. However, the associations were only observed in the Caucasian group and further research, using different cohorts, is needed to further explore this in different ethnic populations.

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